5-1994

# Uptake, Absorption, and Adsorption Kinetics of Ferrous and Ferric Iron in Iron-Replete and Iron-Deficient Rats

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# UPTAKE, ABSORPTION, AND ADSORPTION KINETICS OF FERROUS AND FERRIC IRON IN IRON-REPLETE AND IRON-DEFICIENT RATS

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1994

## ACKNOWLEDGMENTS

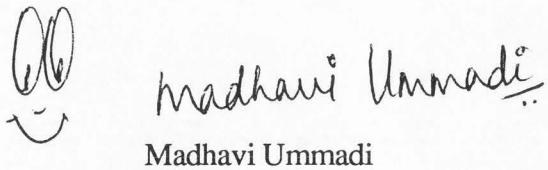
I would like to express my sincere appreciation to my major professor, Dr. Charles E. Carpenter, for his constant guidance and support throughout my master's program, and also for his helpful suggestions and advice during the prepartion of my thesis.

My deepest sense of gratitude is extended to Dr. Deloy G. Hendricks for his constant encouragement, patience, guidance and help through all aspects of my life.

I would also like to express my sincere appreciation to Dr. LeGrande Ellis for his helpful assistance and willingness to serve on my committee.

Thanks to Dr. Deloy Hendricks and my fellow graduate students for their words of encouragement and concern, which have enabled me to persevere throughout my course of study. Special thanks to my dearest cousin Ritu and my friend Hrushi for their constant love and words of encouragement.

I am also grateful to my beloved parents, Shri. U. Lakshmiah and Smt. U. Vijayalakshmi, for showing their constant love, moral support, and appreciation all through my life. My utmost love and appreciation to my uncle and aunt (Shri. U. Pattabhi Ramiah and Smt. U. Sowbhagyavathi), for without their help, I wouldn't be here in the U.S.A. I am grateful to the rest of my family for their support and encouragement while I completed this endeavor.



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# ABSTRACT

Uptake, Absorption, and Adsorption Kinetics of Ferrous and Ferric Iron in Iron-replete and Iron-deficient Rats

by

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Utah State University, 1994

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Various concentrations of ferrous and ferric iron solutions were held at room temperature for 60 min before they were assayed for ferrous iron, which may be unstable due to oxidation. The ferrous and ferric solutions (in pH 2 HCl) were maintained as such for 60 min without the use of chelators. There was no significant oxidation of ferrous iron. Also, four different levels of each ferrous and ferric iron were injected into proximal duodenal loops of rat intestine and uptake was determined at four different time intervals. Two iron-replete rats were assigned to each of the treatments. The in situ experiments showed that iron was taken up rapidly from pH 2.0 solutions of ferrous and ferric iron. Maximum amount of iron was taken up in the first 10 min. Uptake of ferrous iron was significantly greater ($p < 0.05$) than uptake of ferric iron, and there were significant differences in total uptake among the four iron levels used.

Uptake, absorption, and adsorption kinetics of both ferrous and ferric iron were determined in situ for both iron-replete and iron-deficient rats. Deficiency caused greater uptake and absorption, confirming a biological adaptation of these processes. Both uptake

and absorption were greater for ferrous than for ferric iron and were possibly taken up by different pathways or by a ferrous-ferric pathway with preference for ferrous. Uptake and absorption kinetics were biphasic for both ferrous and ferric iron. The first phase demonstrated saturation kinetics and was followed by a nonsaturable phase at higher concentrations of luminal iron. Iron deficiency altered the uptake and absorption kinetics of ferrous and ferric iron, but not always in a similar manner, suggesting that ferrous and ferric iron were each taken up by a separate pathway. Indications were that enhanced absorption during deficiency was largely due to adaptation of ferric uptake. Iron adsorption was directly proportional to luminal iron concentration, but it was greater for ferric than for ferrous, possibly due to charge interactions. Iron deficiency caused increased adsorption of both ferrous and ferric iron, supporting the notion that adsorption acts to maintain iron in a form available for uptake.

(65 pages)

# CHAPTER 1

# INTRODUCTION

## LITERATURE REVIEW

Movement of iron from the intestinal lumen, across the epithelial cells of the digestive tract and into the circulation, is defined as absorption. In spite of an empirical understanding of factors that regulate iron absorption, relatively little is known about the specific mechanisms involved with the intestinal absorptive pathway. Iron absorption occurs primarily in the duodenum and the most proximal part of the small intestine (Conrad et al., 1964). Absorption can be divided into two distinct steps involving uptake from the intestinal lumen into the mucosal cells followed by transfer of a portion of the internalized iron across the mucosal cell and into the circulation. It is generally accepted that the two forms of dietary iron, heme and nonheme, share a common pathway for mucosal transport, but are taken up by different pathways. Heme iron is taken up as an intact porphyrin complex. Inorganic iron is then freed from porphyrin by an enzyme-catalyzed reaction and transported across the mucosa. In contrast, uptake of nonheme iron is not well understood. For example, it is unclear whether the two forms of nonheme iron, ferrous and ferric, are taken up by the same mechanism or by discrete mechanisms. Neither possibility has been discounted during the past 30 years due to lack of techniques which would allow determination of ferrous and ferric uptake under identical conditions. This study established an in situ procedure for estimation of iron uptake kinetics in rats (Chapter 2). This procedure was used to determine the in vivo uptake kinetics of ferrous and ferric iron in both iron-replete and iron-deficient rats (Chapter 3).

***Nonheme iron absorption.*** Iron absorption occurs mostly in the proximal small intestine, where the mucosa remains attuned to current body requirements of iron (Brown

1977, Conrad and Crosby 1963, Conrad et al. 1964, Wheby et al. 1964). Studies show that absorption of iron is a process that exhibits biphasic kinetics (Charlton et al. 1965, Conrad and Crosby 1963, Johnson et al. 1983, Wheby et al. 1964, Wheby and Crosby 1963). An initial rapid period of absorption begins within the first ten seconds of iron reaching the mucosal surface, and lasts for approximately an hour. This is followed by a period (12-24 h) where absorption occurs at a much slower rate.

During the early period of rapid absorption, non-heme iron is absorbed as a result of two processes operating simultaneously. The first step is iron uptake from the lumen. This is followed by a transport step where all or a portion of the internalized iron is transferred into the body. Although these steps are closely integrated to accomplish absorption of iron, each is observed to be independent and specific in its function (Carpenter and Mahoney 1992, Edwards and Bannerman 1970, Edwards and Hoke 1972, Manis 1971). The remainder of the review will focus on the uptake stage of absorption.

***Uptake of iron.*** Perhaps due to the difficulties in presenting ferric iron in soluble form, most early reports indicated that uptake of inorganic iron was only as the ferrous form. However, as previously reviewed (Conrad et al. 1987, Bezkorovainy 1989, Marx and Aisen 1981, Valberg et al. 1983), it is now clear that both ferrous and ferric iron are equally well absorbed from the intestinal lumen provided they are chelated by suitable ligands and presented in soluble forms. For example, the bioavailability of iron as ferrous ascorbate or ferric polymaltose has been found to be quantitatively the same when measured in either rats or humans (Jacobs et al. 1979, Jacobs et al. 1984, Johnson and Jacobs 1990, ). Even high molecular weight ferric hydroxide polymers are well absorbed in vivo. Both ferrous and ferric iron have been shown to follow quantitatively similar uptake in vitro (Berner et al. 1986).

The controversy is no longer if ferric iron is absorbed, but how it is absorbed as compared to ferrous iron. To paraphrase similar quotes from Flanagan (1989) and Peters

et al. (1988), "The precise specificity of intestinal uptake mechanism(s) for ferrous and ferric iron in vivo is not clear." Based on literature, it is not possible to verify whether uptake of ferrous and ferric iron is via the same mechanism or by discrete mechanisms.

***Uptake of iron in a ferrous form.*** According to Hellbock and Saltman (1967), the intestinal regulatory system requires reduction of the ferric iron to a much more soluble ferrous form before it is absorbed. In vivo studies using in situ administration of iron into isolated gut segments of rat small intestine have indicated that reduction of ferric iron to ferrous is necessary for intestinal absorption (Wollenberg and Rummel 1987). It can, however, be argued that soluble factors necessary for ferric absorption were washed away by saline. The subsequent return of iron absorption by adding ascorbic acid would naturally be expected due to reduction of ferric to ferrous and absorption by a ferrous pathway. This would bypass the necessity of any ferric transport.

Addition of the ferrous-chelator, ferrozine, to mixtures of ferric iron chelates completely inhibited absorption of ferric iron. Since ferrozine-bound iron is unavailable for absorption, it was elucidated that ferric iron must be reduced to ferrous for absorption. It was also seen that the lumen of the intestine contained endogenous reducing activity which, when removed by a saline wash, prevented absorption of ferric chelates. Absorption of the ferric chelates could be returned to normal levels by administering ascorbic acid with the chelates. Additionally, including the ferroxidase ceruloplasm to the ferric iron chelate mixtures has been observed to prevent iron absorption (Barrand et al. 1990).

Additional support for uptake of ferrous iron comes from in vitro studies. Manis and Schacter (1962) found that both the ferrous and ferric form of iron were transferred from the mucosal to the serosal surface of everted gut sacs of rat. However, serosal transfer of ferrous iron was greater than was ferric iron. Marx and Aisen (1981), using isolated brush border membrane vesicles from rabbit, reported binding of both ferrous

and ferric iron to the membrane, but only ferrous crossed the membrane into an osmotically active space. Ferrous uptake exhibited saturation kinetics but was unhindered by heating of the membrane. They concluded that ferrous iron was transported across the membrane by simple diffusion against an apparent concentration gradient. Muir et al. (1984) performed additional studies using brush-border vesicles isolated from iron-replete and iron-deficient mice and reported that ferrous transport across the membrane was carrier mediated, which is the first step for uptake. Ferric iron was not observed to cross the membrane, although it did bind to the membrane. Membrane transport of ferrous iron as well as binding of ferric and ferrous forms was biologically mediated since all increased in vesicles from iron-deficient animals.

***Uptake of iron in a ferric form.*** In contrast to the earlier theory of ferrous iron entering the mucosa lies the possibility that iron is taken up in both ferrous and ferric form by separate pathways. Both low molecular weight ferric iron ($FeNO_3$) and soluble high molecular weight ferric hydroxide polymers were well absorbed in vivo (Berner et al. 1986, Berner et al. 1985). Geisser and Müller (1987) have shown that the pharmokinetic behavior of the ferrous and ferric iron absorption and distribution are totally dissimilar. Results of their in vivo experiments using rats suggested that ferrous absorption is by passive diffusion and is not hindered by any feedback mechanism. In contrast, absorption of ferric iron appeared to be via an active transport mechanism or rate determining ligand exchange. In their model, ferrous iron transfer across mucosal membranes was limited by membrane surface area and concentration gradient, whereas ferric iron uptake was controlled by biologic feedback. This model is consistent with ferrous iron, but is not consistent with soluble ferric chelates. Additional in vivo evidence for distinct, separately regulated mechanisms for the uptake of ferrous and ferric iron comes from studies of mouse duodenum. Hypoxia was found to produce distinct

effects on ferrous and ferric iron uptake; ferric iron uptake was greatly enhanced, but ferrous iron uptake was unaffected (Simpson et al. 1986a, Simpson et al. 1986b).

Further supporting evidence for the possible uptake of iron as ferrous and ferric comes from recent in vitro studies. Both ferrous and ferric iron have been reported to cross the brush border membrane of isolated vesicles and duodenal cells (Simpson and Peters 1984, Simpson and Peters 1986a, Simpson and Peters 1986b, Simpson et al. 1985). In these studies, the transport of ferric iron across the brush border membrane was found to be directly dependent on the concentration of free ferric iron in solution. It was suggested that the failure of other researchers to observe membrane transport of ferric iron was due to their use of high molar ratios of ferric chelators that would minimize free ferric iron. The uptake of ferrous and ferric iron was found to markedly differ in both rate and response to various inhibitors. The difference in response to inhibitors may reflect the different chemical properties of ligands used to stabilize the two ions rather than the direct effects on uptake mechanisms. Both mechanisms were correlated in their sensitivity to metabolic inhibitors, exhibition of saturation kinetics, and adaptive response to iron requirements. The exact manner in which iron uptake is coupled to metabolic activity is unclear.

Raja et al. (1989) have suggested that ferric uptake by mouse duodenal fragments is dependent on the brush border membrane potential. Iron uptake by vesicles was quantitatively and qualitatively similar to in vivo uptake for ferrous iron (Simpson and Peters 1986a), but only qualitatively similar for ferric iron (Simpson and Peters 1986b). However, Raja et al. (1987a), using isolated duodenal fragments instead of vesicles, found that uptake of ferric iron in vitro to be both quantitatively and qualitatively correlated with in vivo iron uptake. The discrepancy in quantitative ferric uptake between duodenal cells as compared to brush border vesicles was later explained by evidence suggesting the presence of two pathways for ferric iron uptake in duodenal cells,

one of which is lost during purification of the vesicles (Raja et al. 1987a, Simpson and Peters 1986a ). Since rates of ferric and ferrous uptake are similar in vivo (Simpson and Peters 1986a), but the rates of uptake of vesicles is at least 25 times faster for ferrous than for ferric (Simpson and Peters 1984, Simpson et al. 1985), the ferric uptake pathway that is lost during purification of vesicles is quantitatively the most important. Ferric uptake by the vesicles may be due to mediation by nonesterified fatty acids that complex with iron, thereby forming neutral lipophilic complexes of Fe n+(fatty acid)n that can transverse the membrane (Simpson et al. 1988). The role of fatty acid-mediated uptake in vivo has not been determined. This pathway cannot account for the saturation kinetics or rates of uptake in vivo, but it may be important in mediating iron transport across various organelle membranes (Peters et al. 1988).

Another pathway for ferric iron uptake by the mucosa has been proposed by Huebers et al. (1983). They believe transferrin, a ferric-binding protein, is secreted into the intestinal lumen where it can complex with ferric. The intact complex is then transferred into the mucosal cell via receptor-mediated transport. Inside the cell, iron is released from the transferrin, and iron-free transferrin returns to the brush border to be recycled. The stomach hormone gastrin has been suggested to play a role in the transferrin model of ferric iron uptake. Gastrin can bind both ferric iron and apotransferrin (Baldwin et al. 1986, Longano et al. 1988). It has been proposed that gastrin initially binds dietary ferric iron in the stomach. After the gastrin-ferric complex enters the small intestine, the gastrin binds apotransferrin, and ferric iron is transferred to transferrin. Since gastrin does not bind saturated transferrin, the latter is released to deliver iron to the mucosa.

Other research has produced results inconsistent with the transferrin model for ferric absorption. Results of Simpson and Peters (1986b) suggest that receptor-mediated endocytosis of transferrin is not a significant mechanism of ferric iron uptake. In their in

situ studies on absorption from ligated mouse gut segments transferrin iron was a much poorer enhancer of ferric absorption than was nitriloacetate-chelated iron. This is contrary to expected results if there is a ferric transport mechanism specific for transferrin. Although receptors for transferrin have been localized in mucosal cells, they have not been found in the brush border membrane where they could effect transferrin transport from the intestinal lumen (Banerjie et al. 1986). Transferrin receptors were found solely in the serosal membrane where they increased in number during iron deficiency. It is, therefore, likely that the function of transferrin receptors is to: 1) increase iron transport out of the mucosal cell, or 2) increase iron absorption from blood transferrin, thereby providing iron for intestinal maintenance. Another problem is defining the origin of the secreted transferrin. Luminal transferrin has been suggested to come from the goblet cells of the mucosa or in the bile from the liver. However, these sources have been questioned. Izdera et al. (1986) found rat mucosal cells to be devoid of transferrin messenger RNA. Schumann et al. (1986) demonstrated that although transferrin is found in bile, its iron-binding capacity is exceeded by bile's iron content. Thus bile transferrin would be saturated with iron and could not react with dietary iron.

Recent in vitro experiments using isolated brush border membranes from both experimental animals and humans have confirmed the presence of distinct, saturable, and the rate-limited processes for the uptake of each ferrous and ferric iron (Cox and Peters 1979, Muir et al. 1984, Simpson and Peters 1984, Simpson and Peters 1986a, Simpson and Peters 1986b).

***Measurement of iron uptake.*** In situ procedures can be considered the best method for determination of uptake kinetics, as compared to other procedures. Using an in vivo procedure requiring oral dosing is not a viable option because of inadequate control over concentration of the iron forms entering the intestine; concentration of iron forms within the intestine would be unknown and variable as a result of variability in

rates of gastric emptying, dilution of iron concentrations by gastric fluids, and interconversion of iron forms within the stomach. Determining the uptake kinetics using the in vitro procedures, using isolated membrane vesicles, is not a preferred technique since uptake pathways for ferric may be lost during preparation of vesicles (Raja et al. 1987a, Raja et al. 1987b, Simpson and Peters 1986a).

In situ procedures typically require the intestinal segment to be washed with isotonic saline prior to measuring radiotracer. Although this is a simple procedure, it may overestimate the iron that has actually entered the mucosal cells. Johnson et al. (1983) have demonstrated that much of the iron associated with washed intestine is merely adsorbed or nonspecifically bound. This adsorbed iron is likely to be bound to the mucus layer of the intestine (Conrad et al. 1991, Quarterman 1987, Wein and Van Campen 1991). It is possible to measure only the internalized iron by determining the iron bound to the intercellular iron-binding proteins, ferritin and transferrin. However, it is technically demanding and time consuming (Conrad et al. 1987b, Huebers et al. 1990, Savin and Cook 1980).

Radiolabel techniques have gained acceptance as a powerful tool for measurement of iron bioavailability from foods. These techniques have been used extensively to estimate iron-bioavailability in both humans and animals. Various methods have been used to measure iron absorption from foods. The chemical balance technique measures iron absorption directly from the whole diet, i.e., using difference of iron content between food and feces. Alternatively, the absorption of food iron may be assessed by measuring the degree of retention of an iron isotope given to the subjects in extrinsically or intrinsically tagged food (O'Dell 1984, Smith 1983). Intrinsic labeling consists of addition of the isotope to the nutrient culture solution in which foods or organisms are grown. This culture solution is then incorporated biosynthetically under normal physiological conditions into the animal tissues. Studies have provided some information

about the bioavailability of iron from specific foods (Moore et al. 1944), but this kind of approach has its limitations (Björn-Rasmussen and Hallberg 1974; Cook et al. 1972). It is not a valid measurement of iron absorption from whole diet. And it is also expensive and time consuming in preparing intrinsically labeled foods (Consaul and Lee 1983).

Extrinsic labeling is a technique in iron bioavailability studies that has become popular owing to its simplicity, convenience, accuracy, and low cost over the intrinsic labeling techniques (Buchowski et al. 1991, Consaul and Lee 1983, Cook 1983) Extrinsic radioactive iron behaves in a manner entirely analogous to the element intrinsic to most foods as pointed out by several investigators (Hallberg 1984, O'Dell 1984, Van Campen 1983). A small amount of radiotracer, added as a soluble iron salt, exchanges completely with the nonheme pool (Van Campen 1983). The extrinsic radioactive isotope added to the food comes to equilibrium with all pools of the element in the food (O'Dell 1984).

The tracer used for extrinsic tagging in our experiments was inorganic $^{59}Fe$. The half life of this radioactive isotope is 45.6 days. Nonheme iron absorption from $^{59}Fe$ extrinsically labeled foods was studied by Buchowski et al. (1991) using rat bioassay to mimick the human model. Although the general physiology of the rat does not exactly imitate the human absorption of iron, iron absorption by human beings and rat ranked similarly (Cook et al. 1973). This persisting similarity under a variety of conditions adds support to the use of an animal model in the preliminary assessment of iron absorption (Forbes et al. 1989). Calculations on the results of dose-response studies of non-heme and heme iron absorption indicate that the kinetic processes for iron absorption in human volunteers are similar to those of a rat (Manis and Schacter 1962).

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# CHAPTER 2

# DEVELOPMENT OF A PROCEDURE TO MEASURE UPTAKE OF FERROUS AND FERRIC IRON[1]

## ABSTRACT

Various concentrations of ferrous and ferric iron solutions were held at room temperature for 60 min and were assayed for ferrous iron, which may be unstable due to oxidation. The ferrous and ferric solutions (in pH 2 HCl) were maintained as such for 60 min, without the use of chelators. There was minimal oxidation of ferrous iron. Four different levels of ferrous and ferric iron were injected into ligated duodenal loops of rat intestine, and the retention was quantitated at four different time intervals. Two iron-replete rats were assigned to each of the treatments. The in situ experiments showed that iron was taken up rapidly from pH 2.0 solutions of ferrous and ferric iron. Maximum amount of iron was taken up in the first ten min. Uptake of ferrous was significantly greater ($p < 0.05$) than uptake of ferric iron. Total uptake was a direct function of the iron concentration used.

## INTRODUCTION

Use of an in situ procedure was decided upon as the best methodology for successful determination of uptake kinetics. Uptake kinetics using an in vivo procedure requiring oral dosing was determined not to be a viable option because of inadequate control over concentration of the iron forms entering the intestine, which would be unknown and variable as a result of variability in rates of gastric emptying, dilution of

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[1] Coauthored by Madhavi Ummadi and Charles E. Carpenter.

iron concentrations by gastric fluids, and interconversion of iron forms within the stomach. Determining the uptake kinetics using in vitro procedures involving isolated membrane vesicles was not a preferred technique since uptake pathways for ferric may be lost during preparation of vesicles (Raja et al. 1987a, Raja et al. 1987b, Simpson et al. 1986).

It is clear that solubility of iron in the intraluminal medium of the gastrointestinal tract is a prerequisite for its absorption. Iron in aqueous solution exists only in two stable valence states, ferrous and ferric, that differ in their solubilities. Ferrous iron can exist from an acidic to a neutral pH, but it is easily oxidized to ferric iron as the pH increases. Ferric iron can exist in solution only in acid environments such as found in the stomach. Solubility of ferric iron decreases at pH values greater than three because the sparingly soluble ferric hydroxides are formed. Ferric iron has to be either chelated to a suitable ligand or kept at acidic pH in order to maintain its solubility (Forth and Rummel 1973, Hellbock and Saltman 1967, Valberg et al. 1983).

This study established an in situ procedure for estimation of iron uptake kinetics. This procedure used ligated duodenal loops of rat intestine and required that the iron be administered in slightly acidic solutions so that the iron remained in solution and remained in the form in which it was administered. This avoided the use of chelators to maintain ferric solubility, which could cause problems during in situ absorption studies due to the influence of the chelators on iron uptake (Clydesdale 1983).

## MATERIALS AND METHODS

***Experimental design.*** In experiment 1, various iron solutions were held at room temperature for 60 min and then assayed for ferrous iron, which may be unstable due to oxidation. Solutions of ferrous and ferric iron were made by diluting 1000 ppm respective stock solutions with 0.001 N HCl. A 3 x 4 factorial design was used with three

different levels of ferrous iron (100, 200, and 400 μg/mL) and four different levels of ferric iron (0, 100, 200, and 400 μg/mL).

In experiment 2, four different levels each of ferrous and ferric iron were injected into ligated duodenal loops of rat intestine and uptake was determined at four different time intervals. The levels of iron used in the uptake experiments were 10, 25, 50, and 100 μg/mL for each ferrous and ferric iron. Uptake was determined at 10, 20, 30, and 40 min. This gave a 2 x 4 x 4 factorial design (2 iron forms X 4 different iron levels X 4 time intervals). Two rats were assigned to each of the 32 treatments. One day before the in situ absorption experiment, all the animals were weighed and their hemoglobin levels were determined. The rats were then assigned for each treatment to balance Hb levels and body weight.

***Animals.*** Weanling male Sprague-Dawley rats that were 21 days old were used in this study (Simonson Laboratories, Inc., Gilroy, CA). Rats were individually housed in stainless steel cages with wire mesh bottoms and fronts. The animal room was temperature-controlled at 32°C and was on a 12-h light:dark cycle. The animals were fed casein-based purified diet (Mahoney et al. 1979), except that the beef suet was replaced by corn oil (72.3 g/kg). The diet contained adequate levels of iron to establish iron-replete status in the animals. The iron-supplemented diet contained 35 mg iron/kg diet (Appendix C). The rats were allowed free access to the diet and deionized water for 50 days. Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

***In situ uptake procedure.*** Male albino rats weighing about 150-200 gms were deprived of food overnight, but not deprived of water. Water was removed 2-3 h before the experiment. Rats were anesthetized by giving them an intraperitoneal injection of sodium pentobarbital (4 mg/100 gms body weight) (Anthony Products Co., Arcadia, CA). The anesthetized rats were then laparatomized, and a 10-cm segment of the small

intestine starting from the pylorus was ligated with a cotton string, and a previously prepared standard iron test solution (0.5 ml) tagged with radiolabeled iron (0.5 μCi) was injected into the loops. The $^{59}FeCl_3$ was used to tag ferric and $^{59}FeSO_4$ to tag ferrous iron (Du Pont, NEN Products, Boston, MA). Care was taken not to occlude observable vessels, and then the abdomen was closed with Michel clips. Animals were kept at 32°C for the duration of the experiment. After a preselected time interval, the rats were exsanguinated by decapitation and duplicate blood samples (200 μl) were collected in a test tube for counting.

***Analytical.*** Radioactivity of $^{59}Fe$ in the blood and intestinal segment was measured using a gamma counter (Hewlet Packard Auto-Gamma Model 2000 Series, Meriden, CT). Standards were run each time for each iron level administered to correct for decay and counting efficiency of the machine. The amount of the $^{59}Fe$ that was absorbed into the blood was calculated using the factor of 0.067 ml blood/g body weight (Kim et al. 1993). Total uptake was calculated by combining the values for iron absorbed into the blood and iron associated with the intestinal segment. Ferrous iron was determined spectrophotometrically using Ferrozine color reagent (3-2(pridyl)-5,6 diphenyl-1,2,4-triazone-p,p'-disulphonic acid, Aldrich Chemical Co., Milwaukee, WI) (Carter 1971), a ferrous-specific chromogen. Hemoglobin concentrations were measured colorimetrically using the cyanmethemoglobin method of Crosby et al. (1954).

***Statistical analysis.*** Data were analyzed statistically by ANOVA using STATISTICA (Stat Soft Inc., Tulsa, OK).

## RESULTS AND DISCUSSION

The in situ absorption procedure required that the iron be administered in slightly acidic solutions so that the iron both remained in solution and remained in whichever form it was administered without the use of any chelators. The acid environment ensured

the solubility of ferric iron, but oxidation of ferrous iron was of special concern. However, in experiment 1, ferrous iron was maintained by itself, and in the presence of ferric iron, over a wide range of ferrous concentrations (100-400 ppm). The overall recovery of ferrous iron averaged 97% after 60 min and was not affected ($p > 0.05$) by the level of ferric iron added. This was sufficient time for the proposed in situ absorption experiments to be performed and suggested only minimal oxidation of ferrous iron. Chelators were not used because future experiments require both the iron forms to be administered simultaneously. Although chelators have been successfully used to administer single iron forms, either ferric or ferrous, many factors complicate their use, such as appropriate chelator for each iron form, binding constants for each chelator to iron form, changes in redox potential of the chelated iron forms, molar ratios of chelator to iron of each form, etc. Because most of these parameters are not well understood for even one chelator and one iron form, there appeared to be little chance of obtaining well-characterized mixtures using chelators.

In experiment 2, in situ absorption of ferrous and ferric iron were determined in iron-replete rats at various time intervals. There was measurable uptake at all times and iron levels. Maximum amount of iron was taken up in the first 10 min, with no significant difference between time intervals. Uptake of ferrous was significantly greater ($p < 0.05$) than uptake of ferric iron. For each ferrous and ferric iron, there were also significant differences in total uptake among the four iron levels used. Amount of injected ferrous and ferric iron is plotted against iron uptake at 10 min in Figure 2-1.

The use of the nonphysiological pH of 2 for the injected solutions may be questioned. However, pH of the mucus layer and luminal cell membrane, where uptake occurs, has been shown to be maintained at neutrality for at least 60 min, independent of the pH of the luminal bulk solution (Flemstrom and Kivilaakso 1983). Thus, the pH at the luminal membrane should not be altered from neutrality during the 10 min in situ

experiments reported here. Confirmation that the luminal pH is being maintained at neutrality was based on the absence of visually identifiable tissue damage and bleeding that would occur upon acidification of the luminal membrane (Flemström and Kivilaakso 1983).

This remarkable capacity to provide the stable environment at the luminal cell membrane has been attributed to alkanization of an unstirred water layer by intestinal secretion of $HCO_3^-$ (Flemström and Kivilaakso 1983; Ryu and Grim 1982) and delayed migration of $H^+$ across the mucus layer which coats the cells (Takeuchi et al. 1983, Williams and Turnberg 1980). The thickness of the luminal environment that is maintained at normal pH has been shown to be considerably greater than the thickness of the mucus layer (Flemström and Kivilaakso 1983). Thus, any mineral-binding properties of the mucus (Conrad et al. 1991, Quarterman 1987, Wein and Van Campen 1991) should not be altered and should not affect iron uptake.

Much experimental evidence documents that injecting solutions of pH 2 into intestine does not alter the pH of the mucus layer and the luminal cell membrane, even during extended time periods. Thus, the process of mineral uptake by the luminal cell membrane should not be altered by the pH of the injected solutions used in the short duration, in situ experiments reported here.

In conclusion, it was possible to maintain ferrous and ferric iron in pH 2.0 solutions for 60 min without the use of chelators. Both ferrous and ferric iron were absorbed in situ from pH 2.0 solutions with substantial uptake occurring during the first 10 min.

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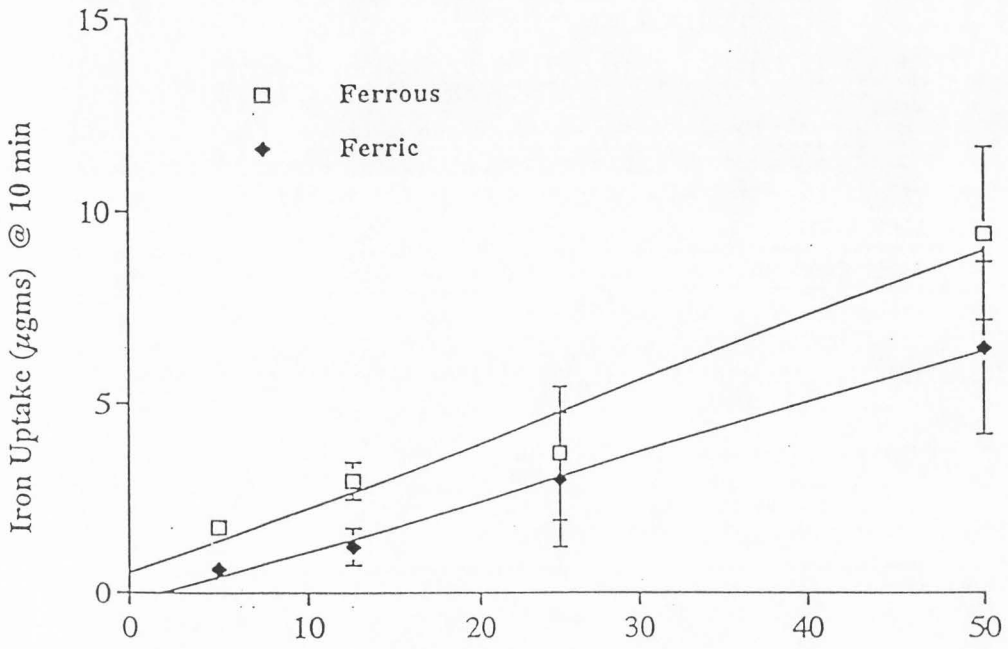


**Figure 2-1** Total uptake of ferrous and ferric iron in iron-replete rats. Error bars represent ± 1 S.D

# CHAPTER 3

# ADSORPTION, UPTAKE, AND ABSORPTION KINETICS OF FERROUS AND FERRIC IRON IN IRON-REPLETE AND IRON-DEFICIENT RATS[1]

## ABSTRACT

Adsorption, uptake, and absorption kinetics of both ferrous and ferric iron were determined in situ for both iron-replete and iron-deficient rats. Iron adsorption to the intestine was greatest ($p < 0.05$) in iron-replete rats, which is consistent with the hypothesis that adsorption is a protective mechanism against absorption of excess iron. Adsorption was directly proportional to the concentration of luminal iron, but more ferric iron was adsorbed, possibly due to charge interactions. Deficiency caused greater uptake and absorption of iron, which is in agreement with the current understanding of iron metabolism. Both uptake and absorption were greater for ferrous iron than for ferric iron. Uptake and absorption kinetics were biphasic for both types of iron. The first phase was characterized by saturation kinetics and was followed by a nonsaturable phase at higher concentrations of luminal iron. Iron deficiency also increased the maximal velocity of uptake and absorption about ten-fold for ferric iron, but less than two-fold for ferrous iron, suggesting that ferrous and ferric iron are each taken up by a separate pathway.

## INTRODUCTION

Absorption, the movement of iron from the intestinal lumen across the epithelial cells of the digestive tract and into the circulation, occurs largely from the proximal intestine in a two-step process involving the rapid uptake of iron from the intestinal

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lumen into the mucosa and the transfer of a portion of this iron across the mucosal cell and serosal membrane into the circulation. Free ferrous iron or chelated ferric iron have been typically used in experiments measuring uptake and absorption kinetics (Geisser and Muller 1987, Huebers et al. 1990, Nathanson et al. 1985, Srai et al. 1988, Thomson et al. 1971, Thomson and Valberg 1971). However, chelators influence iron uptake (Clydesdale 1983), which precluded a direct comparison of ferrous and ferric iron kinetics.

In this research, we developed an in situ procedure that made it possible to administer both ferrous and ferric iron without the use of chelators. The administration of either form of iron in a solution of 0.01 N HCl meant that iron remained in solution, and oxidation of ferrous iron was delayed. Both ferrous and ferric iron, individually and in mixtures, were stable for at least an hour in 0.01 N HCl, ample time to complete the in situ experiments. Both ferrous or ferric iron were absorbed from these acidic solutions. Acidic solutions should not alter the uptake process since pH at the luminal cell membrane is independent of the bulk solution in the lumen (Flemström and Kivilaakso 1983). We employed this in situ technique to determine the adsorption, uptake, and absorption kinetics of free ferrous and ferric iron in both iron-replete and iron-deficient rats.

## MATERIALS AND METHODS

***Experimental design.*** Uptake kinetics of both ferrous and ferric iron in both iron-replete and iron-deficient rats were determined in situ in four treatments (2 iron forms x 2 levels of iron status). Nine different concentration levels of iron were tested using nine rats per concentration. Concentrations were 10, 20, 40, 60, 80, 100, 150, 200, and 300 mg iron/L for the iron-replete rats and 10, 80, 150, 300, 400, 500, 600, 800, and 1000 mg/L for the iron-deficient rats using nine rats per each concentration. The

concentrations for iron-deficient rats were higher than for iron-replete rats because of their increased absorption that accompanies deficiency. Rats were purchased as weanlings (3 weeks old) and were fed casein-based purified diets (Mahoney et al. 1979), except that the beef suet was replaced by corn oil (72.3g/kg). Rats were maintained on the basal diet (~15 mg iron/kg diet) or the basal diet plus added iron (35 mg FeCl3 added per kg diet) for 50 days in order to establish an iron-deficient or iron-replete status, respectively. One day before the in situ absorption experiment, all the animals were weighed and Hb levels determined by using the cyanomethemoglobin method (Crosby et al. 1954). The rats were then assigned to nine groups balanced for Hb levels and body weight. Each group was assigned to a ferric or ferrous iron concentration used in the uptake studies. Each day, for 10 days, one rat from each group received its assigned iron concentration in the in situ absorption procedure.

Nine rats per day appeared to be a practical limit for the in situ uptake procedure, and it was not logistically possible to simultaneously accommodate all the rats required for the in situ experiment (2 levels of iron status x 2 iron forms x 9 different iron concentrations x 9 rats per treatment = 324 rats) due to the rapid growth and physiological changes that were occurring in these young, growing rats. Therefore, the rats were acquired in four separate batches, and each batch was assigned to one of the four treatments. The first two batches were assigned to the iron-replete group, one batch for estimating ferric uptake kinetics and the other batch for ferrous uptake kinetics. Similarly, the latter two batches were assigned to the iron-deficient group, one batch for estimating ferric uptake kinetics and the other for ferrous uptake kinetics.

To reduce the chance that there were differences in iron absorption between batches of rats, 10 rats were randomly selected from each batch to serve as a batch-reference. Rats in this group received the iron-supplemented diet. Rats in the batch-reference groups were gavaged with a dose of radiolabeled ferrous iron to determine

absorption into the blood and liver. Samples were taken at 24 h when initial clearance of absorbed iron was complete and values were stable (Geisser and Muller 1987). Iron absorption in the batch-reference groups was evaluated to determine whether or not there were inherent differences in iron absorption between batches.

We also determined the effect of low-iron and iron-supplemented diets on iron absorption. Upon arrival, 10 rats were randomly selected from each batch to serve as a diet reference group. These received the same diet as rats used for the kinetic studies. Iron absorption in the batch-reference groups was determined at the initiation of the in situ experiments by gavaging them with radiolabeled ferrous iron and determining 24-h absorption into the blood and liver.

***Animals.*** Sprague-Dawley rats were purchased as weanlings (21 days old) from Simonson Laboratories, Inc. (Gilroy, CA). The principles of laboratory animal care as promulgated by the National Society of Medical Research were observed. Rats were individually housed in stainless steel cages with wire mesh bottoms and fronts. The animal room was temperature-controlled to 32°C and was programmed with a 12-h light:dark cycle. The rats were allowed free access to the assigned diets and deionized water for 50 days. Rats were grown to 150-200 g on the assigned diets prior to determination of uptake and absorption kinetics. Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

***In situ uptake procedure.*** Food, but not water, was withheld from the rats overnight. Water was removed 2-3 h before the experiment. Rats were anesthetized with an intraperitoneal injection of sodium pentabarbital (4 mg/100 gms body weight) (Anthony Products Co., Arcadia, CA). The anesthetized rats were then laparatomized, and a 10-cm segment of the small intestine, starting from the pylorus, was ligated with a cotton string, taking care not to occlude visible vessels. Iron solution (0.5 ml) tagged with radiolabelled iron (18.5 kBq) was injected into the loops. $^{59}FeCl_3$ was used to tag

ferric iron and $^{59}FeSO_4$ to tag ferrous iron (NEN Products, Boston, MA). The abdomen was closed with Michel clips, and the animals were kept at 32°C for the duration of the experiment. After 10 min, the rats were exsanguinated by decapitation and triplicate blood samples (200 µl) were collected. The ligated segment of intestine was then removed, the contents were drained, and the lumen was flushed with isotonic saline. The lumen of the segment was immediately filled with about 2 ml of warm 3% agar solution, clamped shut with hemostats, and allowed to solidify on ice for about 30 sec. The segment was then transferred to a clean sheet of paper, cut open lengthwise, and the agar cast with the adhering mucus was collected in a test tube for counting (Wein and Van Campen 1991).

The radioactivity in blood, liver, intestinal segment, and agar cast was measured to determine iron adsorption, uptake, and absorption. Adsorbed iron was estimated from the radioiron content of the intestinal mucosal layer removed with the agar cast. The total amount of iron absorbed into the blood was calculated using the factor 0.067 ml blood/g body weight (Kim et al. 1993), based on radiotracer found in the blood and liver. The amount of iron that had taken up was calculated based on radiotracer found in the blood and liver plus radiotracer found within the intestinal segment.

***Analytical.*** Radioactivity of samples was measured using a gamma counter (Hewlet Packard Auto-Gamma Model 2000 Series, Meriden, CT). Standards were run with all samples to correct for decay and counting efficiency of the machine.

***Statistical analysis.*** Total adsorption, uptake, or absorption of iron was compared by ANOVA in a randomized block design with iron concentrations nested within treatments (STATISTICA software for Macintosh, Stat Soft Inc., Tulsa, OK). Kinetic parameters were estimated by using nonlinear least squares fitting rates of iron uptake and absorption vs concentration of administered iron (JMP statistical analysis program, SAS Institute Inc., Cary, NC). Rates were determined by dividing total adsorption,

uptake, or absorption of iron by 10 min, the duration of the in situ experiment. Rates were fit to either the Michelis-Menton equation (saturable kinetics) or the general linear equation (nonsaturable kinetics).

## RESULTS & DISCUSSION

***In situ determination of iron uptake, absorption, and adsorption.*** Both ferrous and ferric iron were taken up and absorbed from the solutions (pH 2) used to administer the iron, which agrees with previous observations (Berner et al. 1985, Berner et al. 1986, Forth and Rummel 1973, Wein and Van Campen 1991). The acidic pH increased the solubility of ferric iron and delayed oxidation of ferrous iron. Injecting the acidic solutions does not alter the pH of the mucus layer and the luminal cell membrane, where uptake occurs, even when such a solution is injected for an extended time (Flemström and Kivilaakso 1983), and should not alter iron uptake by the luminal cell membrane during the short experiments reported here. Tissue damage and bleeding are quickly visible upon acidification of the luminal membrane (Flemström and Kivilaakso 1983). No tissue degradation was observed in these studies, an indication that the luminal pH was being maintained at neutrality. The ability to maintain a neutral pH at the luminal cell membrane may be due to alkanization of an unstirred water layer by intestinal secretion of $HCO_3^-$ (Flemström and Kivilaakso 1983; Ryu and Grim 1982) and delayed migration of $H^+$ across the mucus layer which coats the cells (Takeuchi et al. 1983, Williams and Turnberg 1980). Neutral pH is maintained through the mucus layer (Flemström and Kivilaakso 1983) which means that any iron-binding properties of the mucus (Conrad et al. 1991, Quarterman 1987, Wein and Van Campen 1991) would not be altered and would not affect iron uptake.

Only internalized iron was measured in the intestinal segments. Adsorbed iron was removed from the intestinal segments before counting using the technique of

Quarterman (1987). The technique involves injecting a warm agar solution into the intestinal segment, chilling the segment, splitting it lengthwise, and separating the agar cast (containing the mucus layer) from the mucosa. In situ uptake procedures typically require washing of the intestinal segment with isotonic saline prior to measuring radioiron content. However, washing does not remove all adsorbed iron (Johnson et al. 1983) and may lead to an overestimation of iron uptake.

***Iron absorption by batch- and diet-reference rats.*** In vivo absorptions of ferrous iron did not differ ($p > 0.05$) between batch-reference groups of rats, which confirmed that no inherent differnces in iron absorption were present between batches. For diet-reference rats, hemoglobin levels (g hemoglobin/L; mean$\pm$SD) were lower ($p < 0.05$) in rats receiving the low-iron diet (83$\pm$12) than in rats receiving the iron-supplemented diet (154$\pm$10). In vivo absorption (% of iron dose absorbed; mean$\pm$SD) was greater for rats receiving the low-iron diet (49$\pm$9) than in rats receiving the iron-supplemented diet (2.5$\pm$1), which confirmed that the dietary regimens altered iron status and elicited a biological adaptation of iron absorption. Dietary regimen had an identical effect on hemoglobin levels of rats used for the in situ experiments (83$\pm$11 for batches receiving the low-iron diet and 154$\pm$10 for batches receiving the iron-supplemented diet).

***Total adsorption, uptake, and absorption.*** Table 1 gives the comaprisons for the total amount of iron processeed during the in situ experiments. Iron adsorption decreased during deficiency, which indicated that adsorption is biologically regulated. The decrease in adsorption was accompanied by increases in uptake and absorption (discussed later), which is consistent with the hypothesis that adsorption protects against excess absorption during periods of iron sufficiency (Wein and Van Campen 1991), but contradicts the hypothesis that adsorption enhances absorption (Conrad et al. 1991, Quarterman 1987). Iron-replete and iron-deficient rats adsorbed more ferric iron than ferrous iron, perhaps due to charge-mediated interactions (ferric iron has greater charge than ferrous iron) with

components of the mucus, such as mucin. Mucin binds iron in a pH-dependent manner consistent with binding due to charge interactions (Conrad et al. 1991). Uptake and absorption of both ferrous and ferric iron were highest in the iron-deficient rats, which is in agreement with the current understanding of iron metabolism. Uptake absorption of ferrous iron exceeded uptake and absorption of ferric iron in both iron-replete and iron-deficient rats. The greater uptake of ferrous may be due to separate pathways for ferrous and ferric iron uptake (Nichols et al. 1992; Simpson et al. 1986) or may be due to an increased transport of ferrous by a ferrous-ferric pathway (Barrand et al. 1990, Wollenberg and Rummel 1987).

***Kinetics of adsorption, uptake, and absorption.*** The rates of iron adsorption to the intestinal mucous layer were linear functions of luminal iron concentration with 0 for axis intercepts. Thus, the previously discussed differences in total absorption also reflect differences in slope. In contrast, uptake and absorption kinetics were biphasic (Figures 3-1through 3-4). The kinetic parameters for uptake and absorption were estimated by using nonlinear least squares fitting of rates vs luminal iron concentration. These parameters are given in Table 2 and were used to draw the curves shown in Figures 3-1through 3-4. At the lower concentrations of luminal iron, uptake and absorption had the saturation kinetics typical of an enzyme-mediated process. At the higher concentrations of luminal iron, uptake and adsorption kinetics were concentration-dependent, characteristic of diffusion-mediated processes. These results are consistent with previous reports of biphasic kinetics of uptake and absorption (Geisser and Muller 1987, Huebers et al. 1990, Nathanson et al. 1985, Srai et al. 1988, Thomson et al. 1971, Thomson and Valberg 1971, Wheby et al. 1964). The saturable and unsaturable phases of uptake and absorption occurred over similar luminal iron concentrations, which is consistent with the hypothesis that uptake is the rate-limiting step of absorption (Cox and Peters 1979, Nathanson et al. 1985). However, maximal rates of uptake exceeded maximal rates of absorption, which

indicated that absorption was limited at some step occurring after uptake. Rates of iron absorption may reflect the competition between the rates of incorporation into mucosal storage (Carpenter and Mahoney 1992).

Iron deficiency extended the saturatable phases of both uptake and absorption to include higher concentrations of luminal iron and altered the kinetics ($K_m$ and $V_{max}$). However, the kinetic changes differed for each ferrous and ferric iron. The most notable difference was the ten-fold increase in the $V_{max}$ for ferric uptake and absorption as compared to less than a two-fold increase in the $V_{max}$ for ferrous uptake and absorption. Iron deficiency increased the $K_m$ for uptake and absorption of ferric iron but decreased the $K_m$ for uptake and absorption of ferrous iron. The different responses of ferrous and ferric suggest that uptake of each occurred by separate pathways and that enhanced absorption during deficiency was largely due to adaptation of ferric uptake. Similarly, adaptation of ferric uptake system is associated with the increased iron absorption caused by hypoxia in mice (Simpson et al. 1986). Slopes of the unsaturatable phases of uptake and absorption were similar during iron deficiency and iron repletion, which indicated that this phase is under no biological control. Since concentration-dependent uptake occurred at only high concentrations of luminal iron, this type of uptake is probably not important in the normal absorption of iron from food but may play a role with pharmocolagical doses received with iron supplements (Geisser and Muller 1987).

In summary, iron adsorption kinetics were concentration-dependent, but not characteristic of an enzyme-mediated process. Adsorption underwent adaptation in response to iron status; adsorption increased with iron sufficiency, which may protect against excess iron absorption. More ferric iron than ferrous iron was adsorbed, perhaps due to charge interaction with components of the intestinal mucus. Kinetics of iron uptake were biphasic. At the lower concentrations of luminal iron, uptake and absorption kinetics were concentration-dependent, characteristic of diffusion-mediated processes.

Uptake and absorption also underwent adaptation in response to iron status; deficiency caused total uptake and absorption to increase, extended the saturatable phases of uptake and absorption to higher concentrations of luminal iron, and increased maximum rates of uptake and absorption. However, iron deficiency increased the maximum velocity of uptake and absorption about ten-fold for ferric iron, but less than a two-fold for ferrous iron, suggesting that ferrous and ferric iron were taken up by separate pathways.

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**TABLE 1**

***Effects of iron form and iron status on amount of iron processed by rats in situ*[1]**

| Main effect and experimental block | Process | | |
|---|---|---|---|
| | Uptake | Absorption | Adsorption |
| Iron form | | | |
| Iron replete rats | ferrous > ferric | ferrous > ferric | ferrous < ferric |
| Iron-deficient rats | ferrous > ferric | ferrous > ferric | ferrous < ferric |
| Iron status | | | |
| Ferrous iron | replete < deficient | replete < deficient | replete > deficient |
| Ferric iron | replete < deficient | replete < deficient | replete > deficient |

[1]Comparisons were made using ANOVA on the blocked data for all common levels of injected iron. Significance was at $p < 0.05$.

**TABLE 2**

***Kinetic parameters for in situ iron uptake and aborption by rats*** [1]

| Iron status of rat[2] and iron form | Saturable phase | | | Nonsaturatable phase |
|---|---|---|---|---|
| | $K_m$ (mg/L) | $V_{max}$ (μg/min) | Conc. range (mg/L) | Slope (L/min x$10^3$) |
| UPTAKE | | | | |
| Iron-replete rats | | | | |
| ferrous | 481 | 1.3 | 10-200 | 3.9 |
| ferric | 76 | 0.2 | 10-100 | 2.7 |
| Iron-deficient rats | | | | |
| ferrous | 114 | 2.2 | 10-400 | 4.7 |
| ferric | 233 | 2.0 | 10-400 | 4.2 |
| ABSORPTION | | | | |
| Iron-replete rats | | | | |
| Ferrous | 696 | 1.0 | 10-200 | 2.4 |
| Ferric | 26 | 0.1 | 10-80 | 2.0 |
| Iron-deficient rats | | | | |
| Ferrous | 56 | 1.4 | 10-400 | 2.8 |
| Ferric | 180 | 1.4 | 10-400 | 3.1 |

[1]Kinetic parameters were estimated by using nonlinear least squares fitting of the data to the Michaelis-Menton equation for the saturatable phase or to the general linear equation for the non-saturable phase.

[2]Iron status in g hemoglobin/L (mean ±SD) was 154±10 for the iron-replete rats and 83±11 for iron-deficient rats.

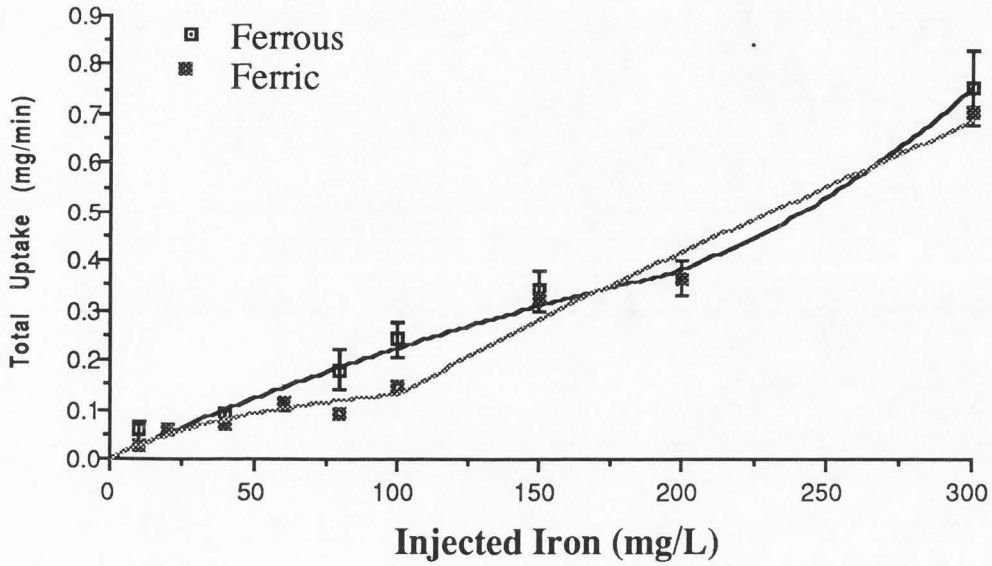


**Figure 3-1**. Uptake of ferrous and ferric iron by iron-replete rats. Error bars represent ± 1 S.E. The curves connecting the data points were generated using the parameters given in table 2.

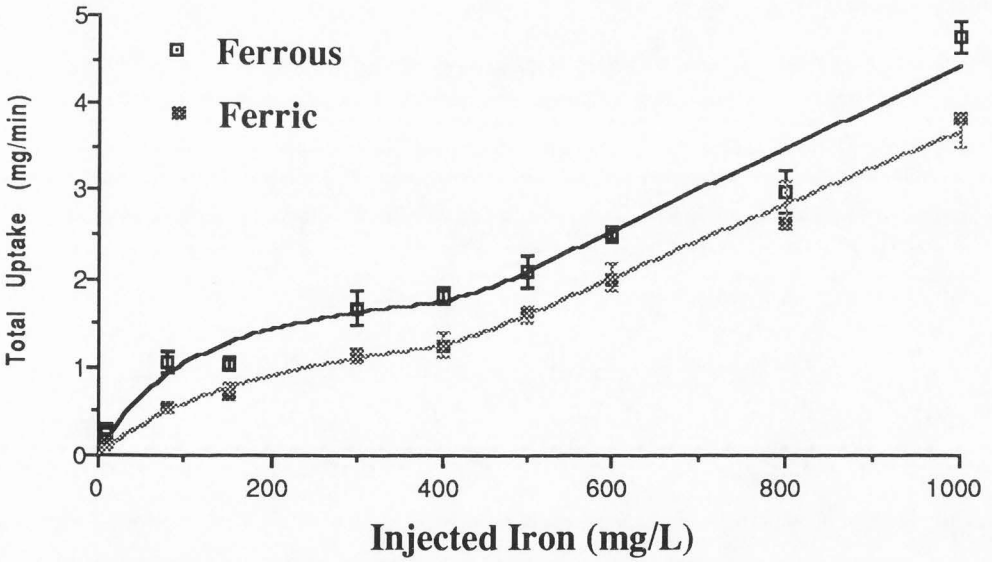


**Figure 3-2**. Uptake of ferrous and ferric iron by iron-deficient rats. Error bars represent ± 1 S.E. The curves connecting the data points were generated using the parameters given in table 2.

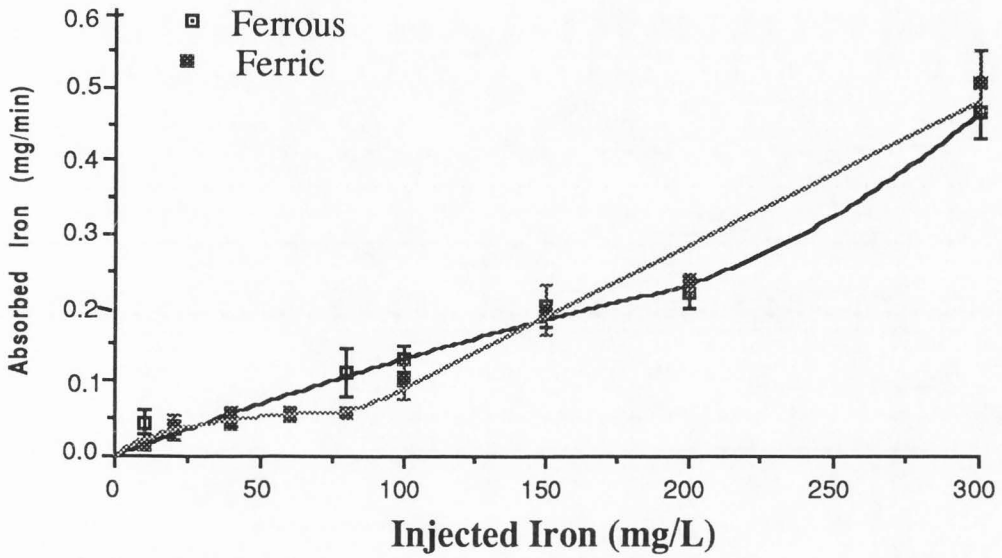


**Figure 3-3**. Absorption of ferrous and ferric iron by iron-replete rats. Error bars represent ± 1 S.E. The curves connecting the data points were generated using the parameters in table 2.

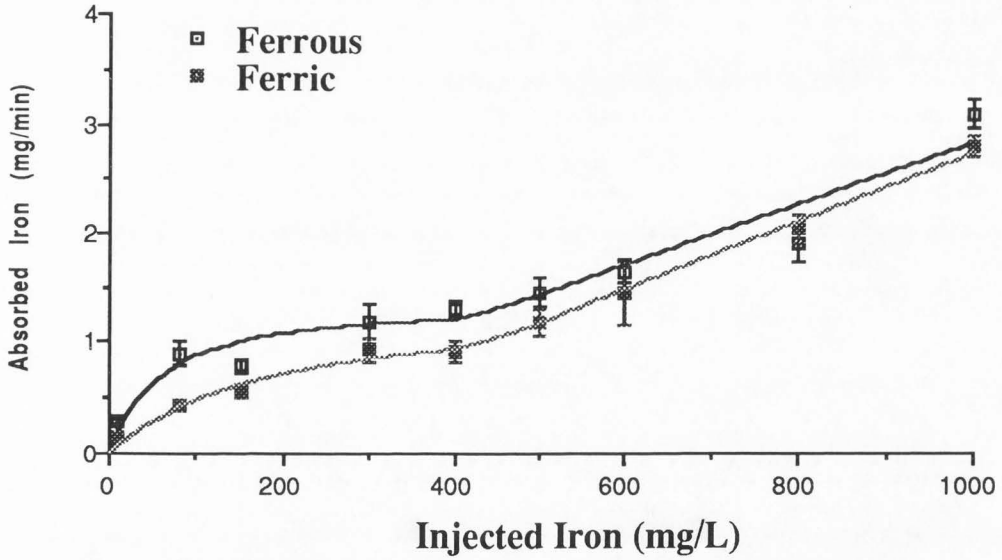


**Figure 3-4**. Absorption of ferrous and ferric iron in iron-deficient rats. Error bars represent ± 1 S.E. The curves connecting the data points were generated using the parameters given in table 2.

# APPENDICES

## APPENDIX A

Diet composition (g/kg)

| Ingredient | Basal diet (iron-deficient) | Iron-supplemented diet |
|---|---|---|
| Casein | 198 | 198 |
| Corn oil | 100 | 100 |
| Cellulose | 50 | 50 |
| $NaH_2PO_4$ | 26.9 | 26.9 |
| $CaCO_3$ | 17 | 17 |
| Vitamin mixture[a] | 20 | 20 |
| Mineral mixture[b] | 11.6 | 11.6 |
| Iron added with ferrous sulfate (mg/kg) | | 35 |
| Dextrose | 576.5 | 576.5 |

a: Ingredients in the Vitamin mixture (g/kg) (Nutrition Biochemicals Corp., Cleveland, OH).

Vitamin A concentrate (500,000 I. U./g)-1.8, Vitamin D concentrate (850,000 I. U./g)-0.125, Niacin-4.25, Riboflavin-1.0, Pyridoxine hydrochloride-1.0, Thiamin hydrochloride-1.0, Vitamin $B_{12}$ (mg/kg)-1.35, Ascorbic acid-45.0, Calcium pantothenate-3.0, Biotin (mg/kg)-20.0, Folic acid (mg/kg)-90.0, Alpha tocopherol (250 I. U./g)-22.0, Inositol-5.0, Choline chloride-75.0, Meandione-2.25, p-Aminobenzoic acid-5.0.

b: Ingredients of the Mineral mixture (g/kg)

Potassium chloride-296.7, Magnesium carbonate-121.0, Magnesium sulfate-12.7, Cobalt chloride ($CoCl_2.6H_2O$)-0.7, Copper sulfate ($CuSO_4.7H_2O$)-1.6, Potassium iodide-0.8, Sodium molybdate ($Na_2MoO_4.2H_2O$)-0.1, Zinc sulfate ($ZnSO_4.7H_2O$)-28.0, Glucose-538.4.

## APPENDIX B

Cyanmethemoglobin method for determination of hemoglobin concentration

Preparation of Drabkin's reagent

-Sodium bicarbonate, 1 g, Potassium cyanide, 52 mg, and

-Potassium ferricyanide, 198 mg, were weighed, then dissolved and diluted to 1 L in a volumetric flask with demineralized water.

Determination

1) Drabkin's solution, 5 ml, was transferred to test tube.
2) Whole blood, 20 µl was added to the test tube and mixed.
3) The assay mixture was kept in the dark for 10 min.
4) Absorbance was measured at 540 nm and concentration calculated from the standard curve.

Preparation of standard curve

Standards (Fisher Scientific Company, Orangeburg, NY) containing 5.5, 13.1, and 17.1 g hemoglobin/dl were used. Standard, 20 µl, was added to the test tube which contained 5 ml of Drabkin's solution and followed the same procedure as the determination.

# APPENDIX C

Ferrous iron determination using ferrozine

Reagents

-Ferrozine color reagent

Ferrozine color reagent (3-2(pyridyl)-5,6-diphenyl-1,2,4-triazone-p, p'-disulphonic acid, Aldrich Chem. Co., Milwaukee, WI) was dissolved in demineralized water to make 1 mM solution.

-Ammonium acetate (10% solution).

-Ascorbic acid (dissolved into 0.1 N HCl to make 0.02% solution).

-Ferrous sulfate (1000 ppm solution) (0.050 g $FeSO_4$ + 10 ml 0.001 N HCl)

-Ferric chloride (1000 ppm solution) (0.048 g $FeCl_3$ + 10 ml 0.001 N HCl)

Determination

1) Three different levels of ferrous iron (100, 200, and 400 μg/mL) and four different levels of ferric iron (0, 100, 200 and 400 μg/mL) were mixed together and set for 60 min at room temperature.

2) First, 0.05 ml sample was added to 1.25 ml of 0.1 N HCl.

3) Ascorbic acid, 0.02% solution, 1.25 ml, was added only to control sample.

4) One milliliter ammonium acetate solution was added and mixed.

5) Ferrozine color reagent, 1.25 ml, was added and mixed and placed in the dark for 30 min.

6) Demineralized water, 1.25 ml, was added.

7) Absorbance was measured at 562 nm after 30 min.

8) Concentration of a sample was calculated from standard curve.

Preparation of standard curve

Standards were prepared by dilution of 1000 mg/L FeCl3 (Ricca Chemical Co., Arlington, Texas). Absorbance was measured from 0, 2, 4, 6, 8, and 10 mg/L. Same procedure as determination was followed.

## APPENDIX D

Summary data tables

Summary Data of Iron Metabolism in Iron-Deficient rats (µgms±S.E).

| Concentration Ferrous Iron (µg) | Total Uptake | Adsorbed Iron | Intestinal Iron | Absorbed Iron |
|---|---|---|---|---|
| 5 | 3.09±0.19 | 0.29±0.03 | 0.21±0.03 | 2.88±0.17 |
| 40 | 10.52±1.22 | 6.12±0.59 | 1.52±.189 | 9.00±1.15 |
| 75 | 10.36±0.69 | 12.55±0.94 | 2.52±0.31 | 7.84±0.53 |
| 150 | 16.68±2.05 | 33.85±2.11 | 4.75±0.58 | 11.93±1.60 |
| 200 | 17.95±0.89 | 38.15±4.28 | 4.91±0.37 | 13.04±0.76 |
| 250 | 20.79±1.60 | 48.77±5.49 | 6.28±0.58 | 14.51±1.34 |
| 300 | 25.02±0.99 | 47.96±2.97 | 8.57±0.36 | 16.45±1.08 |
| 400 | 29.87±2.34 | 77.93±7.55 | 10.76±0.89 | 19.10±1.67 |
| 500 | 47.60±1.71 | 91.32±6.74 | 16.65±1.43 | 30.95±1.33 |

Summary Data of Iron Metabolism in Iron-Deficient rats (µgms±S.E).

| Concentration Ferric Iron (µg) | Total Uptake | Adsorbed Iron | Intestinal Iron | Absorbed Iron |
|---|---|---|---|---|
| 5 | 1.49±0.10 | 0.24±0.03 | 0.12±0.01 | 1.37±0.13 |
| 40 | 5.34±0.42 | 4.72±0.45 | 0.95±0.08 | 4.40±0.43 |
| 75 | 7.06±0.65 | 14.16±1.63 | 1.49±0.16 | 5.57±0.58 |
| 150 | 11.43±1.38 | 28.90±4.31 | 2.13±0.29 | 9.29±1.13 |
| 200 | 12.34±0.98 | 39.60±4.96 | 3.22±0.50 | 9.12±0.99 |
| 250 | 16.26±1.48 | 66.01±5.69 | 4.43±0.83 | 11.84±1.23 |
| 300 | 19.88±2.53 | 73.97±9.82 | 5.49±0.45 | 14.39±2.85 |
| 400 | 26.26±1.74 | 115.87±9.03 | 5.87±1.01 | 20.39±1.41 |
| 500 | 38.25±1.64 | 136.86±10.72 | 10.22±1.66 | 28.03±0.94 |

Summary Data of Iron Metabolism in Iron-Replete rats (µgms±S.E).

| Concentration Ferrous Iron (µg) | Total Uptake | Adsorbed Iron | Intestinal Iron | Absorbed Iron |
|---|---|---|---|---|
| 5 | 0.59±0.58 | 0.51±0.23 | 0.14±0.06 | 0.45±0.54 |
| 10 | 0.60±0.09 | 1.02±0.19 | 0.24±0.04 | 0.36±0.07 |
| 20 | 0.85±0.17 | 3.14±0.34 | 0.33±0.04 | 0.53±0.13 |
| 30 | 1.11±0.11 | 5.46±0.59 | 0.54±0.07 | 0.57±0.07 |
| 40 | 1.81±0.39 | 7.55±0.55 | 0.70±0.10 | 1.11±0.32 |
| 50 | 2.43±0.36 | 10.45±0.92 | 1.14±0.27 | 1.29±0.17 |
| 75 | 3.39±0.41 | 14.79±1.51 | 1.35±0.24 | 2.04±0.29 |
| 100 | 3.67±0.36 | 23.09±2.49 | 1.42±0.26 | 2.22±2.28 |
| 150 | 7.54±0.74 | 34.41±2.28 | 2.89±0.53 | 4.65±0.37 |

Summary Data of Iron Metabolism in Iron-Replete rats (µgms±S.E).

| Concentration Ferric Iron (µg) | Total Uptake | Adsorbed Iron | Intestinal Iron | Absorbed Iron |
|---|---|---|---|---|
| 5 | 0.29±0.07 | 0.46±0.06 | 0.12±0.03 | 0.16±0.04 |
| 10 | 0.58±0.17 | 1.59±0.27 | 0.18±0.04 | 0.39±0.16 |
| 20 | 0.73±0.14 | 3.79±0.49 | 0.23±0.03 | 0.50±0.14 |
| 30 | 1.11±0.17 | 4.99±0.82 | 0.55±0.10 | 0.56±0.08 |
| 40 | 0.94±0.11 | 7.47±1.18 | 0.36±0.04 | 0.58±0.09 |
| 50 | 1.49±0.27 | 8.01±1.00 | 0.49±0.09 | 1.01±0.23 |
| 75 | 3.24±0.54 | 19.93±1.79 | 1.28±0.36 | 1.96±0.34 |
| 100 | 3.60±0.24 | 29.60±2.29 | 1.27±0.20 | 2.34±0.13 |
| 150 | 7.06±0.66 | 38.93±3.69 | 2.00±0.36 | 5.07±0.42 |

# APPENDIX E

Summary of statistical analysis for in-vitro kinetic experiments

Preliminary Uptake Data (Exp I).
These are the Individual Ferrous and Ferric Iron values (Means and S.D).
'FERROUS'

| STATISTICA GENERAL MANOVA | Means | |
|---|---|---|
| INJECTED | UPTAKE | Valid N |
| 50 | 1.740750 | 8 |
| 125 | 2.964219 | 8 |
| 250 | 3.689063 | 8 |
| 500 | 9.420625 | 8 |
| All Groups | 4.453664 | 32 |

'FERROUS'

| STATISTICA GENERAL MANOVA | Standard Deviations | |
|---|---|---|
| INJECTED | UPTAKE | Valid N |
| 50 | .665898 | 8 |
| 125 | 1.568956 | 8 |
| 250 | 1.936367 | 8 |
| 500 | 2.701442 | 8 |
| All Groups | 3.484231 | 32 |

'FERRIC'

| STATISTICA GENERAL MANOVA | Means | |
|---|---|---|
| INJECTED | UPTAKE | Valid N |
| 50 | .632375 | 8 |
| 125 | 1.225625 | 8 |
| 250 | 2.983438 | 8 |
| 500 | 6.428750 | 8 |
| All Groups | 2.817547 | 32 |

'FERRIC'

| STATISTICA GENERAL MANOVA | Standard Deviations | |
|---|---|---|
| INJECTED | UPTAKE | Valid N |
| 50 | .165124 | 8 |
| 125 | .486908 | 8 |
| 250 | 1.759453 | 8 |
| 500 | 2.262959 | 8 |
| All Groups | 2.678390 | 32 |

Iron stability-data (In-Vitro Experiment) size: 32 * 4 MISS=-9999.
Include all cases

| STATISTICA GENERAL MANOVA | Means | | |
|---|---|---|---|
| FE2ADDED | FE3ADDED | FE2RECOV | %RECOV |
| 100 | •••• | 97.9382 | 97.93825 |
| 200 | •••• | 188.5100 | 94.25500 |
| 400 | •••• | 393.2588 | 98.31469 |

File: iron stability-data size: 32 * 4 MISS=-9999.
Include all cases

| STATISTICA GENERAL MANOVA | MAIN EFFECT: FE2ADDED 1-FE2ADDED, 2-FE3ADDED | | | |
|---|---|---|---|---|
| Depend. Variable | Mean Sqr Effect | Mean Sqr Error | f(df1,2) 2,12 | p-level |
| FE2RECOV | 183119.3 | 41.03326 | 4462.704 | .0000000 |
| %RECOV | 40.3 | 23.38823 | 1.721 | .2202176 |

File: iron stability-data size: 32 * 4 MISS=-9999
Include all cases

| STATISTICA GENERAL MANOVA | MAIN EFFECT: FE3ADDED 1-FE2ADDED, 2-FE3ADDED | | | |
|---|---|---|---|---|
| Depend. Variable | Mean Sqr Effect | Mean Sqr Error | f(df1,2) 3,12 | p-level |
| FE2RECOV | 20.49319 | 41.03326 | .4994287 | .6896368 |
| %RECOV | 3.83039 | 23.38823 | .1637741 | .9187026 |

File: iron stability-data size: 32 * 4 MISS=-9999.
Include all cases

| STATISTICA GENERAL MANOVA | Means | |
|---|---|---|
| FE2ADDED | FE2RECOV | Valid N |
| 100 | 97.9382 | 8 |
| 200 | 188.5100 | 8 |
| 400 | 393.2588 | 8 |
| All Groups | 226.5690 | 24 |

File: iron stability-data size: 32 * 4 MISS=-9999.
Include all cases

| STATISTICA GENERAL MANOVA | Standard Deviations | |
|---|---|---|
| FE2ADDED | FE2RECOV | Valid N |
| 100 | 5.9060 | 8 |
| 200 | 8.3014 | 8 |
| 400 | 5.0926 | 8 |
| All Groups | 126.3444 | 24 |

# APPENDIX F

## Summary of statistical analysis for procedure development

File: Prelim uptake data (Exp II) size: 64 * 6 MISS=-9999.
Include all cases

STATISTICA GENERAL MANOVA — summary of all effects; design: 1-TIME

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | 3 | 10.85726 | 60 | 10.14968 | 1.069714 | .3687750 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

STATISTICA GENERAL MANOVA — Means

| TIME | UPTAKE | Valid N |
|---|---|---|
| 10 | 4.345078 | 16 |
| 20 | 3.163812 | 16 |
| 30 | 2.716281 | 16 |
| 40 | 4.317250 | 16 |
| All Groups | 3.635606 | 64 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

STATISTICA GENERAL MANOVA — Standard Deviations

| TIME | UPTAKE | Valid N |
|---|---|---|
| 10 | 3.744142 | 16 |
| 20 | 2.853342 | 16 |
| 30 | 2.356321 | 16 |
| 40 | 3.589754 | 16 |
| All Groups | 3.191141 | 64 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

| STATISTICA GENERAL MANOVA | summary of all effects; design: 1-IRONFORM | | | | | |
|---|---|---|---|---|---|---|
| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
| 1 | 1 | 42.83007 | 62 | 9.656819 | 4.435215 | .0392607 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

| STATISTICA GENERAL MANOVA | Means | |
|---|---|---|
| IRONFORM | UPTAKE | Valid N |
| FERROUS | 4.453664 | 32 |
| FERRIC | 2.817547 | 32 |
| All Groups | 3.635606 | 64 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

| STATISTICA GENERAL MANOVA | Standard Deviations | |
|---|---|---|
| IRONFORM | UPTAKE | Valid N |
| FERROUS | 3.484231 | 32 |
| FERRIC | 2.678390 | 32 |
| All Groups | 3.191141 | 64 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

STATISTICA summary of all effects; design:
GENERAL 1-INJECTED
MANOVA

| Effect | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | 3 | 143.2392 | 60 | 3.530586 | 40.57095 | .0000000 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

STATISTICA Means
GENERAL
MANOVA

| INJECTED | UPTAKE | Valid N |
|---|---|---|
| 50 | 1.186563 | 16 |
| 125 | 2.094922 | 16 |
| 250 | 3.336250 | 16 |
| 500 | 7.924687 | 16 |
| All Groups | 3.635606 | 64 |

File: Prelim uptake data size: 64 * 6 MISS=-9999.
Include all cases

STATISTICA Standard Deviations
GENERAL
MANOVA

| INJECTED | UPTAKE | Valid N |
|---|---|---|
| 50 | .739765 | 16 |
| 125 | 1.437167 | 16 |
| 250 | 1.824061 | 16 |
| 500 | 2.860497 | 16 |
| All Groups | 3.191141 | 64 |

# APPENDIX G

## Summary of statistical analysis for in-vivo kinetic experiments

```
STATISTICA MANCOVA RESULTS
MANCOVA
STATS

      DESIGN: 2 - way MANOVA, fixed effects
  DEPENDENT: 3 variables:    ABSORPTI AGAR     UPTAKE
    BETWEEN: 1-IRONFORM( 2): FERRIC FERROUS
             2-IRONLEVE( 9):  10  20  40  60  80 100 150 200 300
     WITHIN: NONE
    NESTING: (2)IRONLEVE in  (1)IRONFORM

     SELECTION CONDITION:
     v1='replete'
```

```
size: 324 * 7  MISS=-9999.
Include If: v1='replete'
```

STATISTICA GENERAL MANOVA — Means

| IRONFORM | IRONLEVE | ABSORPTI | AGAR | UPTAKE |
|---|---|---|---|---|
| FERRIC | •••• | 1.395804 | 12.75319 | 2.115821 |
| FERROUS | •••• | 4.217718 | 11.15870 | 2.441624 |

```
size: 324 * 7  MISS=-9999.
Include If: v1='replete'
```

STATISTICA GENERAL MANOVA — MAIN EFFECT: IRONFORM, 1-IRONFORM, 2-IRONLEVE

| Depend. Variable | Mean Sqr Effect | Mean Sqr Error | f(df1,2) 1,144 | p-level |
|---|---|---|---|---|
| ABSORPTI | 322.5096 | 3.03220 | 106.3617 | .0000000 |
| AGAR | 102.9673 | 20.39290 | 5.0492 | .0261584 |
| UPTAKE | 4.2990 | 1.09990 | 3.9085 | .0499517 |

STATISTICA MANOVA STATS — MANCOVA RESULTS

DESIGN: 2 - way MANOVA, fixed effects
DEPENDENT: 3 variables: ABSORPTI AGAR UPTAKE
BETWEEN: 1-IRONFORM( 2): FERRIC FERROUS
2-IRONLEVE( 9): 10 80 150 300 400 500 600 800 ***
WITHIN: NONE
NESTING: (2)IRONLEVE in (1)IRONFORM

SELECTION CONDITION:
v1='deficien'

size: 324 * 7 MISS=-9999.
Include If: v1='deficien'

STATISTICA GENERAL MANOVA — Means

| IRONFORM | IRONLEVE | ABSORPTI | AGAR | UPTAKE |
|---|---|---|---|---|
| FERRIC | **** | 11.59974 | 53.36963 | 15.36813 |
| FERROUS | **** | 13.96610 | 39.65920 | 20.20669 |

size: 324 * 7 MISS=-9999.
Include If: v1='deficien'

STATISTICA GENERAL MANOVA — MAIN EFFECT: IRONFORM
1-IRONFORM, 2-IRONLEVE

| Depend. Variable | Mean Sqr Effect | Mean Sqr Error | f(df1,2) 1,144 | p-level |
|---|---|---|---|---|
| ABSORPTI | 226.785 | 13.7452 | 16.49926 | .0000796 |
| AGAR | 7613.018 | 261.9296 | 29.06513 | .0000003 |
| UPTAKE | 948.174 | 21.5864 | 43.92452 | .0000000 |

These following results are for Part III

```
STATISTICA  MANCOVA RESULTS
MANCOVA
STATS

      DESIGN: 2 - way MANOVA, fixed effects
  DEPENDENT: 3 variables:    ABSORPTI AGAR     UPTAKE
    BETWEEN: 1-STATUS  ( 2): DEFICIEN REPLETE
             2-IRONLEVE( 4):  10  80 150 300
     WITHIN: NONE
    NESTING: (2)IRONLEVE in  (1)STATUS

     SELECTION CONDITION:
     v2='ferric' and v7=10 or v7=80 or v7=150 or v7=300
```

Size: 324 * 7  MISS=-9999.
Include if: v2='ferric' and v7=10 or v7=80 or v7=150 or v7=300

| STATISTICA GENERAL MANOVA | Means | | |
|---|---|---|---|
| STATUS | IRONLEVE | ABSORPTI | AGAR | UPTAKE |
| DEFICIEN | •••• | 6.345242 | 12.59518 | 8.044993 |
| REPLETE | •••• | 1.965392 | 15.50258 | 3.068751 |

size: 324 * 7  MISS=-9999.
Include if: v2='ferric' and v7=10 or v7=80 or v7=150 or v7=300

STATISTICA GENERAL MANOVA — MAIN EFFECT: STATUS; 1-STATUS, 2-IRONLEVE

| Depend. Variable | Mean Sqr Effect | Mean Sqr Error | f(df1,2) 1,118 | p-level |
|---|---|---|---|---|
| ABSORPTI | 552.4729 | 4.93082 | 112.0447 | .0000000 |
| AGAR | 243.4450 | 44.62342 | 5.4555 | .0211946 |
| UPTAKE | 713.1738 | 8.78939 | 81.1403 | .0000000 |

STATISTICA MANCOVA RESULTS
MANCOVA
STATS

DESIGN: 2 - way MANOVA, fixed effects
DEPENDENT: 3 variables: ABSORPTI AGAR UPTAKE
BETWEEN: 1-STATUS ( 2): DEFICIEN REPLETE
2-IRONLEVE( 4): 10 80 150 300
WITHIN: NONE
NESTING: (2)IRONLEVE in (1)STATUS

SELECTION CONDITION:
v2='ferrous' and v7=10 or v7=80 or v7=150 or v7=300

size: 324 * 7 MISS=-9999.
Include if: v2='ferrous' and v7=10 or v7=80 or v7=150 or v7=300

STATISTICA GENERAL MANOVA — Means

| STATUS | IRONLEVE | ABSORPTI | AGAR | UPTAKE |
|---|---|---|---|---|
| DEFICIEN | •••• | 6.723811 | 12.60765 | 8.445007 |
| REPLETE | •••• | 2.038767 | 15.51604 | 3.144799 |

size: 324 * 7 MISS=-9999.
Include if: v2='ferrous' and v7=10 or v7=80 or v7=150 or v7=300

STATISTICA GENERAL MANOVA — MAIN EFFECT: STATUS
1-STATUS, 2-IRONLEVE

| Depend. Variable | Mean Sqr Effect | Mean Sqr Error | f(df1,2) 1,118 | p-level |
|---|---|---|---|---|
| ABSORPTI | 632.1497 | 4.95781 | 127.5058 | .0000000 |
| AGAR | 243.6125 | 44.62462 | 5.4591 | .0211529 |
| UPTAKE | 809.0556 | 8.82217 | 91.7071 | .0000000 |